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The role of hydrophobic active-site residues in substrate specificity and acyl transfer activity of penicillin acylase

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Penicillin acylase of *Escherichia coli* catalyses the hydrolysis and synthesis of β-lactam antibiotics. To study the role of hydrophobic residues in these reactions, we have mutated three active-site phenylalanines. Mutation of αF146, βF24 and βF57 to Tyr, Trp, Ala or Leu yielded mutants that were still capable of hydrolysing the chromogenic substrate 2-nitro-5-[(phenylacetyl)amino]-benzoic acid. Mutations on positions αF146 and βF24 influenced both the hydrolytic and acyl transfer activity. This caused changes in the transferase/hydrolase ratios, ranging from a 40-fold decrease for αF146Y and αF146W to a threefold increase for αF146L and βF24A, using 6-aminopenicillanic acid as the nucleophile. Further analysis of the βF24A mutant showed that it had specificity constants (k_{cat}/K_m) for *p*-hydroxyphenylglycine methyl ester and phenylglycine methyl ester that were similar to the wild-type values, whereas the specificity constants for *p*-hydroxyphenylglycine amide and phenylglycine amide had decreased 10-fold, due to a decreased k_{cat} value. A low amidase activity was also observed for the semisynthetic penicillins amoxicillin and ampicillin and the cephalosporins cefadroxil and cephalexin, for which the k_{cat} values were fivefold to 10-fold lower than the wild-type values. The reduced specificity for the product and the high initial transferase/hydrolase ratio of βF24A resulted in high yields in acyl transfer reactions.

Keywords: site-directed mutagenesis; β-lactam antibiotics; penicillin acylase; substrate specificity; transferase/ hydrolase ratio.

Penicillin acylase (PA) of *Escherichia coli* (EC 3.5.1.11) catalyses the hydrolysis of penicillin G to phenylacetic acid (PAA) and 6-aminopenicillanic acid (6-APA). PA is a heterodimeric periplasmic protein consisting of a small α subunit and a large β subunit, which are formed by processing of a precursor protein. The catalytic nucleophile, a serine, is located at the N-terminus, which is a hallmark of the family of N-terminal nucleophile (Ntn) hydrolases, a class of enzymes which share a common fold around the active site and contain a catalytic serine, cysteine or threonine at the N-terminal position [1]. The reaction mechanism of PA involves the formation of a covalent intermediate and is similar to the well-known mechanism of serine proteases. After attack on the carbonyl carbon of the amide bond by the active-site nucleophile, a covalent acyl-enzyme is formed via a tetrahedral transition state in which the negatively charged oxyanion is stabilized by H-bonds to the oxyanion hole residues βN241 and βA69 [2]. After expulsion of the leaving group from the active site, the acyl-enzyme is deacylated by H_2O or another nucleophile, yielding the final transacylation product and the free enzyme.

PA is used for the production of 6-aminopenicillanic acid (6-APA) by the hydrolysis of penicillin G, but can also be used for the production of semisynthetic β-lactam antibiotics, in which the enzyme catalyses the condensation of an acyl group and a 6-APA molecule [3]. In this condensation reaction, an activated acyl donor, which is in general an amide or a methyl ester of a PAA derivative, acylates the enzyme at the active-site serine, under expulsion of ammonia or methanol. The resulting acyl-enzyme is then deacylated by a β-lactam nucleophile, e.g. 6-APA or 7-desacetoxycephalosporanic acid (7-ADCA), yielding a semisynthetic penicillin or cephalosporin, respectively. Because the production of antibiotics is a kinetically controlled process, with transient accumulation of the desired product, the kinetic parameters of the enzyme determine the yield of the product.

The two most important parameters are (a) the rate of conversion of the substrate (acyl donor) vs. the rate of conversion of the product (antibiotic), and (b) the rate of acyl transfer to a β-lactam nucleophile vs. the rate of acyl transfer to water, expressed as V_s/V_h.

The rate of product hydrolysis (V_P) vs. the rate of hydrolysis of the acyl donor (V_{AD}) at a certain concentration of acyl donor and product is given by [4]:

$$\frac{V_P}{V_{AD}} = \alpha \cdot \frac{[P]}{[AD]} \quad (1)$$

in which α is defined as

$$\alpha = \frac{k_{catP}/K_{mP}}{k_{catAD}/K_{mAD}} \quad (2)$$

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Abbreviations: PA, penicillin acylase; PAA, phenylacetic acid; PAAM, phenylacetamide; PAAOM, phenylacetic acid methyl ester; PG, phenylglycine; (H)PGA, (*p*-hydroxy)-D-phenylglycine amide; (H)PGM, (*p*-hydroxy)-D-phenylglycine methyl ester; 6-APA, 6-aminopenicillanic acid; 7-ADCA, 7-amino desacetoxycephalosporanic acid; NIPAB, 2-nitro-5-[(phenylacetyl)amino]-benzoic acid.

Note: a web site is availble at http://www.chem.rug.nl/biotechnology

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The subscripts AD and P refer to the acyl donor and product, respectively. The specificity (k_{cat}/K_m) of PA for the produced antibiotic is in general 10-fold higher than the specificity for the corresponding acyl donor, leading to high values of α and consequently to significant rates of product hydrolysis, even at relatively high concentrations of the acyl donor [5,6].

In the deacylation reaction of the catalytic cycle, the β-lactam nucleophile and H_2O compete for the acyl-enzyme. The enzyme displays a moderate affinity towards β-lactam nucleophiles, with binding constants of 10–100 mM. Furthermore, the ester bond in the acyl-enzyme is exposed to the solvent. The low affinity for β-lactam nucleophiles and the accessibility of the acyl-enzyme to H_2O cause hydrolysis of the acyl-enzyme and, especially at low nucleophile concentrations, result in low V_s/V_h ratios, reducing the yield of the desired antibiotic.

In the present study, we have used site-directed mutagenesis to investigate which residues and interactions influence the performance of PA in the formation of semisynthetic β-lactam antibiotics. The X-ray structure of the complex of PA with PAA shows that the acyl binding site of PA is made up of several hydrophobic residues from the α and the β subunit (Fig. 1) [2].

Hydrophobic interactions exist between the phenyl rings of PAA and βF24, which are in a stacked conformation. Another phenylalanine, αF146, is located on the opposite side of the binding pocket. It has hydrophobic interactions with PAA and shields the binding site from the solvent. A third phenylalanine, βF57, is located at the bottom of the hydrophobic cleft. The shortest distance between the side chain of βF57 and PAA is 4.7 Å, which is too long for a direct interaction between βF57 and the substrate. However, residue βF57 may be important for maintaining the structure of the binding site given the short distances of 3.5 Å and 3.9 Å between βF57(CZ) and the substrate binding residues βP22(CB) and βF24(CE2), respectively. Changing the acyl binding site by mutagenesis may influence the synthetic capacities of PA in different ways. The relative affinity of the enzyme for the acyl donor compared to the produced antibiotic may be increased, leading to decreased values for α and increased yields. The mutations may also alter the geometry around the ester bond in the acyl-enzyme complex and thereby influence the relative rates of reaction of the acyl-enzyme with different deacylating nucleophiles, leading to changes in the V_s/V_h ratios.

In this paper, we report the effect of modification of the three active-site phenylalanines on the hydrolysis of the chromogenic substrate 2-nitro-5-[(phenylacetyl)amino]-benzoic acid (NIPAB) and the synthesis of β-lactam antibiotics (Fig. 2).

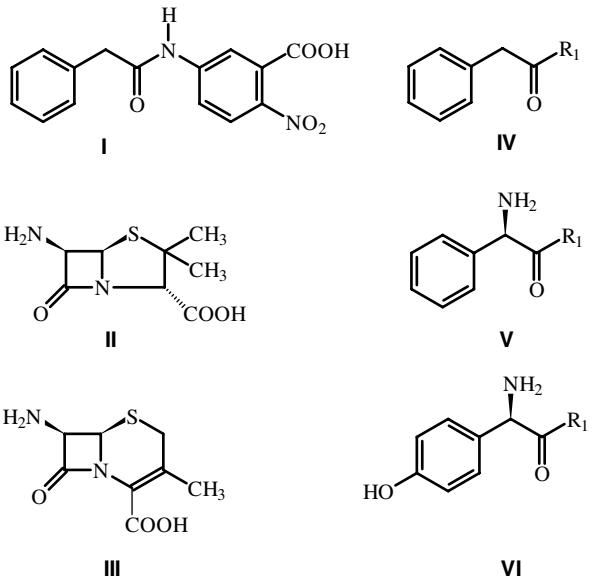

Fig. 2. Substrates of penicillin acylase used in this study. I, NIPAB; nucleophiles: **II**, 6-APA; **III**, 7-ADCA; acyl donors: **IV**, PAAM (R1 = NH2) and PAAOM (R1 = OCH3); **V**, PGA ($R_1 = NH_2$) and PGM ($R_1 = OCH_3$); **VI**, HPGA ($R_1 = NH_2$) and HPGM ($R_1 = OCH_3$).

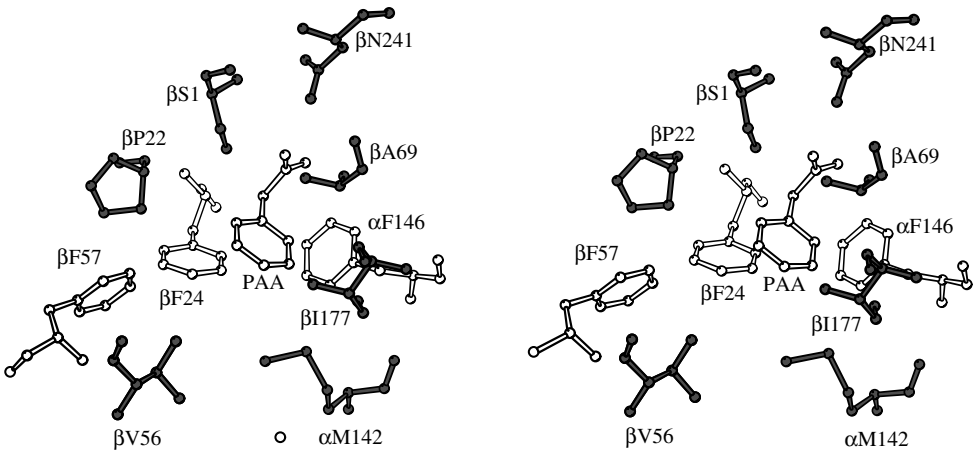

Fig. 1. Stereoview of the active site of penicillin acylase complexed with PAA [2]. The residues that have been mutated in this study, αF146, βF24, βF57 and PAA, are shown in white.

This approach yielded mutants with significantly increased affinity for synthetic acyl donors and with increased potential for transferase reactions.

MATERIALS AND METHODS

Strain and plasmids

Mutants were constructed using the plasmid pEC carrying the PA gene of *E. coli* [7]. For cloning and expression of wild-type and mutant enzymes, *E. coli* HB101 was used as a host.

Site-directed mutations on position αF146 were made as described [7]. For creation of mutants on position βF24 and βF57, fusion PCR was used. Two sets of PCR reactions were carried out using *Pwo* polymerase (Boehringer Mannheim). The first set was carried out using the forward primer BSTXfw 5′-CAGGGAGAACCGGGAAACTA TTG-3′ that anneals upstream of a *BstX*I restriction site in the PA gene, and the reverse primers βF24rv and βF57rv. The βF24rv mutagenic primer was 5′-ATAAGTATACGCAG GCGCATACCAGCCAAACTGCGGGCCATTTAC-3′ and the βF57rv mutagenic primer was 5′-GGAAATC ACACCATTATGACCAAAAACCAGCCCGGGATA GGC-3′. The underlined codons code for βF24 and βF57 and were changed to ATA, CCA, AGC and CAA to introduce Tyr, Trp, Ala and Leu, respectively. The second set of reactions was carried out using the forward primer βF24fw 5′-GGCTGGTATGCGCCTGCGTATACTTAT-3′ or the forward primer βF57fw 5′-GGTCATAATGGTGT GATTTCC-3′, which are complementary to a part of the mutagenic primers, and the reverse primer NHErv, 5′-CAC TCCTGCCAATTTTTGGCCTTC-3′, which anneals downstream of an *Nhe*I site in the gene. Products of both sets of reactions were isolated from agarose gel and used as a template in a third PCR which contained the BSTXfw and NHErv primers. The resulting full-length product was cut with *Nhe*I and *Bst*XI and ligated into the pEC plasmid that was cut with the same enzymes. Ligation products were transformed into $CaCl_2$ competent *E. coli* HB101. All procedures were carried out according to standard protocols [8].

Purifying PE and enzymes

Isolation of periplasmic extracts and purification of the enzymes was carried out as described [7]. Kinetic measurements with βF24L, βF57W, βF57Y, αF146L and αF146A mutants were performed using periplasmic extracts after determination of the concentration of active sites by titration with phenylmethanesulfonyl fluoride as described previously [9]. The total protein concentration was determined according to Bradford [10]. The purity of PA in periplasmic extracts was ≈40%. Because no background activity of β-lactamases, esterases or amidases that could interfere with the kinetic measurements, was observed, these extracts were used for kinetic experiments.

Kinetic analyses

Steady-state kinetic parameters for NIPAB were determined spectrophotometrically as described previously [9] and conversion of other substrates was followed using HPLC [7]. K_i values for PAA and K_m values for substrates were determined by measuring the inhibition on the hydrolysis of NIPAB. The binding constant was calculated using

$$K_{mapp} = K_m \cdot \left(1 + \frac{[I]}{K_i}\right) \quad (3)$$

in which K_{mapp} is the K_m for NIPAB in the presence of inhibitor, [I] the inhibitor concentration, and K_i the inhibition constant or binding constant for the substrate. The k_{cat} was determined separately by measuring the rate of substrate conversion at a concentration of at least 10 times K_m. Conversion of substrates was monitored by HPLC as described previously [7]. Acyl transfer reactions were carried out by mixing enzyme with solutions of acyl donor and nucleophile. Reactions were followed by HPLC and the V_s/V_h ratios were calculated from the initial rates of production of synthesis and hydrolysis product. All enzymatic reactions were carried out at 30 °C at pH 7.0.

Chemicals

NIPAB, PAAOM, D-phenylglycine and *p*-hydroxy-D-phenylglycine were from Sigma-Aldrich. 6-APA, 7-ADCA, HPGA, PGA, HPGM, PGM, cephalexin, amoxicillin, ampicillin, and cefadroxil were obtained from DSM-Gist (the Netherlands).

RESULTS

Activity of site-directed mutants for NIPAB

Three phenylalanines in the acyl-binding site of PA of *E. coli*, βF24, βF57 and αF146, were investigated by site-directed mutagenesis. Each phenylalanine was therefore mutated to Ala, Leu, Trp or Tyr. These mutations may influence the shape and volume of the acyl binding pocket and thereby alter the binding mode and affinity of the enzyme for PAA and derivatives thereof, while maintaining the hydrophobicity of the binding site.

To test the effect of the mutations on the specificity for phenylacetylated substrates, the steady-state kinetic parameters for the hydrolysis of the chromogenic substrate NIPAB and the inhibition constant of the product PAA were determined (Table 1). It appeared that the mutations in all cases led to reduced k_{cat}/K_m values for NIPAB. The effect on the k_{cat} ranged from a 1000-fold decrease for αF146A and αF146L to k_{cat} values of βF24L, βF24Y, βF57L and βF57A that were similar to that of the wild-type enzyme. The K_m for NIPAB had increased in all mutants, except for αF146Y, suggesting that removal of a phenyl group in the hydrophobic binding pocket leads to a reduced affinity for the phenyl group of the substrate. The reduced affinity of the mutants for the phenyl moiety of the substrate was also evident from the twofold to 100-fold increased K_i values for PAA.

From analysis of the k_{cat} values for substrates with the same acyl group and different leaving groups, it was concluded that acylation is the rate-limiting step in the conversion of N-phenylacetylated substrates [9]. Assuming rapid binding of the substrate, it follows that k_{cat} represents the rate of acylation and K_m equals the binding constant of the substrate to the free enzyme. The results then indicate

Table 1. Steady-state kinetic parameters of wild-type and penicillin acylase mutants for the hydrolysis of NIPAB and K_i values for the product phenylacetic acid (PAA). Values represent the mean of 2 independent measurements. Standard deviations are less than 10% from the mean value.

	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}.s^{-1}$)	K_{iPAA} (mM)
WT	16.2	0.015	1080	0.05
βF24A	1.6	0.275	6	1.10
βF24L	36	0.142	248	0.15
βF24W	1.5	0.101	15	0.88
βF24Y	12	0.351	34	1.11
βF57A	17	0.040	425	0.23
βF57L	24	0.049	490	0.11
βF57W	1.6	0.028	57	0.15
βF57Y	0.7	0.320	2.2	4.50
αF146A	0.015	0.055	0.27	0.15
αF146L	0.038	0.025	1.52	0.19
αF146W	1.1	0.032	34.4	3.70
αF146Y	1.6	0.005	320	0.03

Table 2. Kinetic constants of wild-type and penicillin acylase mutants for the synthesis of ampicillin. Reaction conditions were: 15 mM PGA and 30 mM 6-APA, pH 7.0 at 30 °C.

	V_s/V_h	$[Amp]_{max}$ (mM)	$[Amp]_{max}$/[PG]	V^a (% of WT)
WT	1.4	2.2	0.5	100
βF24A	3.0	2.2	1.0	12
βF24L	0.9	1.7	0.3	7
βF24W	0.25	0.4	0.1	4
βF24Y	1.3	1.6	0.4	3
βF57A	1.1	1.9	0.4	42
βF57L	1.6	2.3	0.4	71
βF57W[b]	0.8	–	–	1
βF57Y[b]	1.3	–	–	0.3
αF146A[b]	3.1	–	–	0.6
αF146L	4.2	2.5	1.2	4
αF146W	0.03	0.4	0.015	9
αF146Y	0.033	0.3	0.0216	212

[a] Initial rate of PGA conversion. [b] No reliable $[Amp]_{max}$ could be determined due to the low activity of the enzymes.

that the binding of both the substrate NIPAB and the product PAA are significantly altered by mutating the phenylalanines in the active site suggesting that hydrophobic interactions between the aromatic phenylalanines and the phenyl ring of the substrate play an important role in substrate binding. The large effect of the mutations on the acylation rate indicate that these residues are necessary for correct positioning of the substrate in the active site with respect to the catalytic residues.

Transferase/hydrolase kinetics of the site-directed mutants

From an analysis of the steady-state kinetic parameters for the hydrolysis of NIPAB it was concluded that the mutations led to significantly altered kinetic properties but not to a complete loss of activity of the enzyme. These mutant enzymes were therefore used to study the kinetics of acyl transfer reactions in which 6-APA was used as the acyl acceptor. To this end progress curves of the conversion of phenylglycine amide (PGA) and the formation of phenylglycine (PG) and ampicillin were determined. From these progress curves the V_s/V_h ratio, which represents the relative initial rate of acyl transfer to the β-lactam nucleophile (synthesis) and H_2O (hydrolysis), was obtained. To evaluate the properties of the mutants with respect to production of semisynthetic antibiotics, the maximum product yield $[Amp]_{max}$, the amount of phenylglycine at this point, $[Amp]_{max}$/[PG] and the activity of the mutants, expressed as the initial rate of acyl donor conversion, were also determined (Table 2).

It appeared that the effect of the mutations on the V_s/V_h ratios was much smaller than the effect on the steady-state kinetic parameters for the hydrolysis of NIPAB. In almost all mutants the V_s/V_h ratio was similar to the value of 1.4 that was observed for the wild-type. The largest effect on the V_s/V_h ratio was observed for mutations on positions αF146 and βF24, which caused changes ranging from a 40-fold decrease for the αF146Y and αF146W mutant enzymes to a threefold increase in V_s/V_h for the αF146L, αF146A and βF24A mutant enzymes. Mutating βF57 did not lead to a significant increase or decrease of V_s/V_h. The values for the overall yield $[Amp]_{max}$ and the amount of PG at this point that were obtained using these mutants, were in most cases similar to the wild-type values, in line with the small effects of the mutations on the V_s/V_h ratio.

All mutants, however, showed a decreased activity as indicated by the low initial rates of PGA conversion. This decrease in activity indicates that the mutations influence the rate of acylation by PGA in a similar way as the acylation by NIPAB. A notable exception was the twofold increased activity for PGA of the αF146Y mutant. It appeared that this mutant had a k_{cat} value for PGA that was similar to the wild-type value, and that the increase in activity could be attributed to a K_m of 4.6 mM for PGA of αF146Y, which is almost 10-fold lower than the K_m of 40 mM of the wild-type.

The results indicate that mutating βF57, which is located at the bottom of the binding pocket at 7 Å from the nucleophilic serine, does influence the rate of formation of the acyl-enzyme, as judged by the effect of the mutations on the activity, but does not influence the geometry of the resulting acyl-enzyme with respect to the competing deacylating nucleophiles, as indicated by V_s/V_h values that were similar to wild-type values.

In contrast, mutations at positions αF146 and βF24 affected kinetics for both the acylation and deacylation reactions. These residues are not only located closer to the active-site serine, but may also interact directly with the deacylating nucleophiles 6-APA and H_2O [7].

Steady-state kinetic parameters of βF24A

Two mutants, αF146L and βF24A, combined a higher V_s/V_h with an increased yield of antibiotic and less production of acid compared to the wild-type. However, the activity of both mutants, which is related to the rate of acylation was 10- to 20-fold lower than the wild-type rate. Because acylation of PA by esters is in general faster than acylation by amides [9], these two mutants were employed in

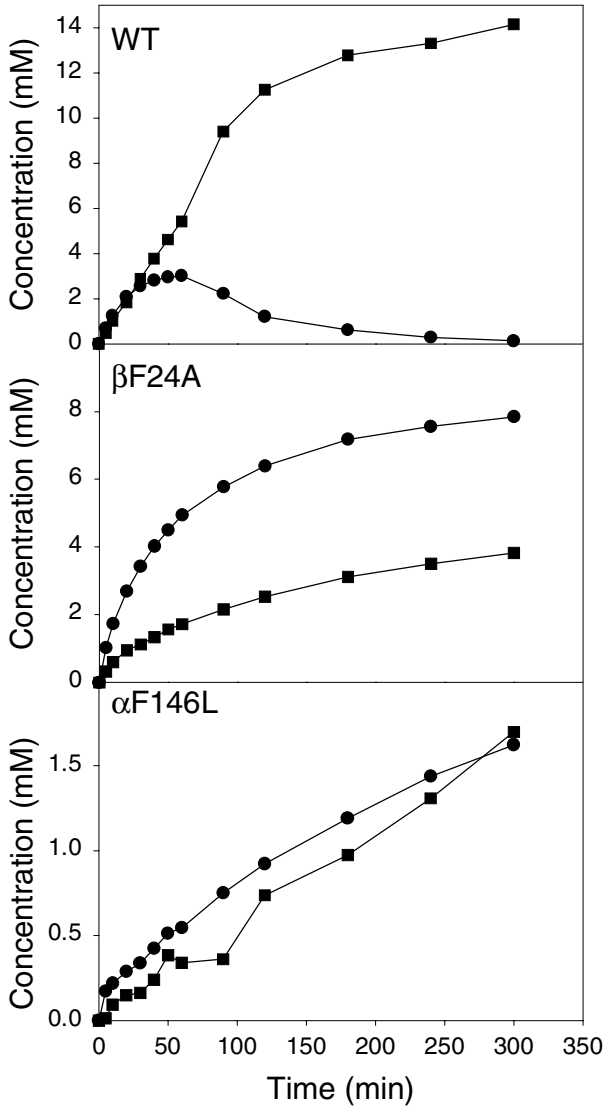

Fig. 3. Kinetically controlled synthesis of ampicillin from 15 mM PGM and 25 mM 6-APA using wild-type penicillin acylase and the βF24A and αF146L mutants (200 nM each). Symbols: (●) ampicillin, (■) PG.

ampicillin synthesis reactions in which the ester (PGM) was used as the acyl donor (Fig. 3).

It appeared that βF24A had the same activity for PGM and an almost threefold increase in $[Amp]_{max}$ and $[Amp]_{max}/[PG]$ compared to the wild-type enzyme. In contrast, the conversion of PGM by αF146L was more than 20-fold slower compared to the wild-type. This shows that in βF24A only the amidase activity was reduced and not the esterase activity, whereas in αF146L both activities had decreased.

The βF24A mutant possessed improved properties for the synthesis of ampicillin, manifested in an increased V_s/V_h and yield and reduced formation of the hydrolysis product. Furthermore, the mutant showed a 20-fold increased inhibition constant for PAA compared to the wild-type. This mutant was therefore investigated in more detail in order to evaluate its applicability in the synthesis of other antibiotics.

First the factor α was determined by measuring the steady-state kinetic parameters of βF24A for the hydrolysis

Table 3. Steady-state kinetic parameters of wild-type penicillin acylase and the βF24A mutant for the hydrolysis of various acyl donors and antibiotics. Values represent the mean of two independent measurements. Standard deviations are less than 10% from the mean value.

	WT			βF24A		
Substrate	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}·s^{-1}$)	k_{cat} (s^{-1})	K_m (mM)	k_{cat}/K_m ($mM^{-1}·s^{-1}$)
PAAM	50	0.20	250	3.9	2	1.94
PAAOM	190	0.16	1187	7.5	2	3.75
PGA	30	40	0.75	2.0	25	0.08
PGM	50	12	4.16	27.7	8.7	3.18
HPGA	10.4	11.7	0.9	2.8	28.7	0.097
HPGM	16.2	12.5	1.3	20	5.9	3.4
Amoxicillin	22	1.1	20	16.1	11.3	1.42
Cephalexin	57	1.2	47.5	9.8	2.8	3.48
Cefadroxil	50	1	50	5.2	0.76	6.87
Ampicillin	30	2.5	12	3.0	1	3

of other relevant synthetic acyl donors and antibiotics (Table 3). It appeared that βF24A had k_{cat} values for HPGM and PGM that were similar to the k_{cat} values of the wild-type, whereas the k_{cat} for the corresponding amides HPGA and PGA was decreased 10-fold compared to the wild-type. A similar reduced k_{cat} value of βF24A was observed for ampicillin, amoxicillin, cefadroxil and cephalexin, which are the antibiotics that can be synthesized from combinations of the two acyl donors and the β-lactam nucleophiles 6-APA and 7-ADCA. In contrast to the increased $k_{cat\ ester}/k_{cat\ amide}$ ratio of βF24A that was observed for synthetic acyl donors, βF24A showed a decreased ratio for the k_{cat} values of the ester/amide pair phenylacetic acid methyl ester (PAAOM) and phenylacetamide (PAAM). Whereas the wild-type had an almost fourfold higher k_{cat} for the ester compared to the amide, the $k_{cat\ ester}/k_{cat\ amide}$ ratio of βF24A was less than 2. The main difference between the synthetic acyl donors and PAA derived substrates is the presence of an NH_2 group on the Cα position. Apparently, interactions between this group and the enzyme influence the esterase/amidase ratio of the enzyme.

The importance of the presence of a Cα-amino group on the substrate for conversion by βF24A was also evident from the K_m values of the mutant enzyme. The K_m values for substrates containing a Cα-amino group were similar and in some cases even lower than the wild-type values, whereas a reduced affinity for the substrates containing a phenylacetyl moiety was observed. The 10-fold increased K_m values for PAAM and PAAOM of βF24A correlate well with the low affinity of βF24A for PAA and NIPAB (Table 1).

In short, the results show that the βF24A mutation leads to an increased esterase/amidase ratio and an increased affinity for Cα substituted synthetic acyl donors relative to PAA.

Transferase/hydrolase kinetics of βF24A PA

The βF24A mutant enzyme had reduced k_{cat}/K_m values for all antibiotics tested compared to the wild-type, whereas k_{cat}/K_m values for the acyl donors PGM and HPGM were

Table 4. Kinetic constants of wild-type penicillin acylase and the βF24A mutant for the synthesis of semisynthetic β-lactam antibiotics. The V_s/V_h ratio was determined by measuring the initial rate of formation of antibiotic and hydrolysis product, using 15 mM of the acyl donor and 30 mM of the α-lactam nucleophile.

			V_s/V_h		α	
Acyl donor	Nucleophile	Product	WT	βF24A	WT	βF24A
HPGM	6-APA	amoxicillin	1.8	3.0	15.4	0.4
HPGA	6-APA	amoxicillin	1.7	3.1	22.2	14.6
PGM	6-APA	ampicillin	1.4	3.1	2.9	0.9
PGA	6-APA	ampicillin	1.4	2.9	16	37.5
HPGM	7-ADCA	cefadroxil	5.2	21	38.5	2.0
HPGA	7-ADCA	cefadroxil	4.6	15	55.6	70.8
PGM	7-ADCA	cephalexin	5.2	18.4	11.4	1.1
PGA	7-ADCA	cephalexin	4.9	15.8	63.3	43.5

similar to wild-type values. This leads to a threefold to 40-fold decrease in the factor α when esters are used as the acyl donor (Eqn 2) (Table 4), indicating that high yields in the synthesis of β-lactam antibiotics could in principle be obtained. A second requirement for efficient synthesis is a high reactivity of the β-lactam nucleophile with the acyl-enzyme. To test the reactivity of 6-APA and 7-ADCA with the βF24A mutant enzyme, initial rates of deacylation, V_s/V_h, were recorded, using the methyl ester or the amide as acyl donor.

It appeared that 6-APA and 7-ADCA were able to efficiently deacylate the phenylglycyl- and *p*-hydroxyphenylglycyl-enzyme of βF24A, as indicated by, respectively, a twofold and fourfold increased V_s/V_h ratio compared to the wild-type (Table 4). The V_s/V_h ratio using 7-ADCA and 6-APA was independent on whether a methyl ester or an amide was used as acyl donor, indicating that the deacylation is not influenced by the leaving group of the acyl donor. Furthermore, it appeared that the presence of a *p*-hydroxy group on the acyl donor did not notably influence relative rates of deacylation of the wild-type and βF24A acyl-enzyme, indicated by similar V_s/V_h ratios for PGM and HPGM with 7-ADCA or 6-APA.

To study the mechanism underlying the increased V_s/V_h ratio of the βF24A mutant, we measured the dependency of V_s/V_h on the concentration of nucleophile [N]. This dependency is hyperbolic and may be described using Eqn (4) [4]:

$$\frac{V_s}{V_h} = \frac{\left(\frac{V_s}{V_h}\right)_{max} \cdot [N]}{K_N + [N]} \qquad (4)$$

In this equation, [N] is the concentration of nucleophile, $(V_s/V_h)_{max}$ represents the maximum V_s/V_h ratio, which is obtained at saturating concentrations of [N], and K_N is the concentration of [N] at which $V_s/V_h = 0.5 \cdot (V_s/V_h)_{max}$. The dependence of V_s/V_h on [N] was measured using PGA as the acyl donor and 6-APA as the nucleophile (Fig. 4). Both for the wild-type and the βF24A mutant enzyme the V_s/V_h levels off to a maximum, indicating that even when the acyl-enzyme is fully saturated with 6-APA, hydrolysis of the acyl enzyme still occurs [11,12].

Fitting Eqn (4) to the data yielded values for K_N of 37 mM and 69 mM and for $(V_s/V_h)_{max}$ of 3 and 10 for the wild-type and βF24A, respectively. This indicates that the improved kinetics of acyl transfer of βF24A are caused by an increased maximum V_s/V_h rather than an increased affinity for 6-APA. The fact that higher V_s/V_h ratios for βF24A were observed at all concentrations of 6-APA, indicates that under a broad range of conditions this mutant is a suitable biocatalyst.

Antibiotic synthesis using βF24A

To study the importance of the kinetic parameters α and V_s/V_h with respect to the yield that can be obtained in a synthesis reaction, progress curves for the production of ampicillin and cephalexin were recorded. Using PGM with 6-APA or 7-ADCA as the nucleophile, a twofold to fourfold higher yield and an increased ratio $[P]_{max}/[PG]$ were obtained in reactions catalysed by the βF24A enzyme, compared to wild-type-catalysed synthesis (Fig. 5).

When the βF24A mutant enzyme was used for the synthesis of the same antibiotics, but with the amide as the acyl donor, for which the βF24A has a higher α than the wild-type, similar yields were obtained as with the wild-type

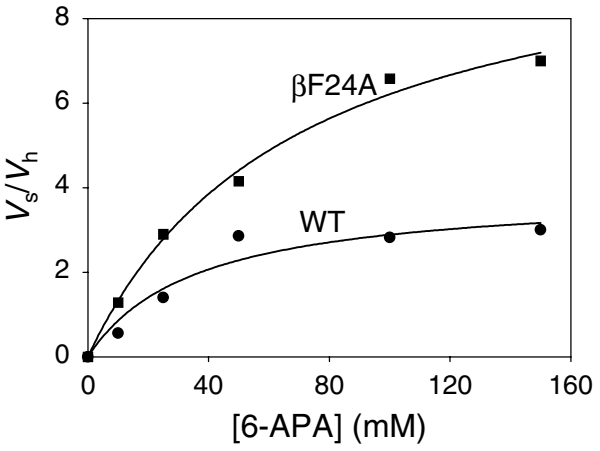

Fig. 4. Dependence of V_s/V_h on the nucleophile concentration, [6-APA], in the synthesis of ampicillin from PGA and 6-APA. The symbols represent experimental data, the line represents the best fit to the data using Eqn (4),derived from the general kinetic scheme for acyl transfer reactions [4]. Parameters used to fit the data were $(V_s/V_h)_{max} = 3.9$ and $K_N = 36$ mM for wild-type and $(V_s/V_h)_{max} = 10.5$ and $K_N = 69$ mM for βF24A. The reactions were carried out with a fixed PGA concentration of 15 mM.

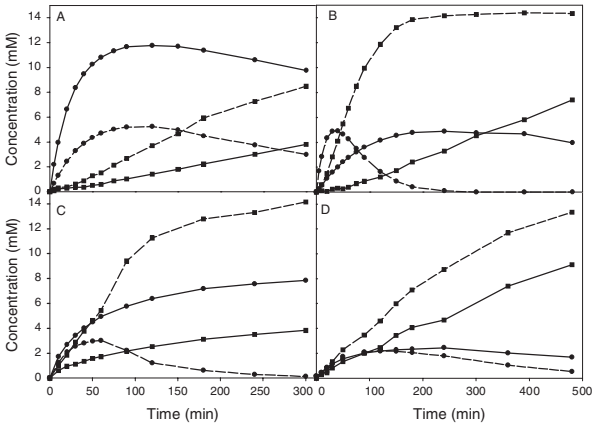

Fig. 5. Kinetically controlled synthesis of ampicillin and cephalexin using wild-type (dashed lines) and the βF24A mutant (solid lines). (A) Cephalexin synthesis from PGM and 7-ADCA; (B) cephalexin synthesis from PGA and 7-ADCA; (C) ampicillin synthesis from PGM and 6-APA; (D) ampicillin synthesis from PGA and 6-APA. Symbols: (●) cephalexin or ampicillin; (■) PG. In all experiments the concentration of the acyl donor was 15 mM and the concentration of nucleophile was 30 mM.

and only a small increase of $[P]_{max}/[PG]$ was observed. In the synthesis of amoxicillin and cefadroxil, using HPGM and HPGA as the acyl donor, similar results were found as for ampicillin and cephalexin synthesis. Thus high yields were obtained with the βF24A mutant enzyme when the ester was used as the acyl donor, whereas no increase in yield compared to the wild-type was observed using the amide as the acyl donor.

These results show that the yields obtained in synthesis correlate well with the steady state kinetic parameters of the enzymes for acylation and deacylation that were determined in independent experiments (Tables 3 and 4). From these data it can be concluded that the highest yields are obtained with the βF24A mutant when the ester is used as the acyl donor, because for this compound both α and V_s/V_h are improved compared to the wild-type. For the amides the βF24A mutant also shows an increased V_s/V_h but this leads to only slightly higher efficiencies compared to the wild-type, because the high value of α of βF24A for synthesis from the amide counteracts the effects of the improved V_s/V_h ratio of the βF24A mutant.

DISCUSSION

The PA-catalysed synthesis of β-lactam antibiotics is a kinetically controlled reaction, which means that the yield of the product from an activated precursor strongly depends on the kinetic constants of the enzyme for acylation and deacylation. In this paper we describe the use of site-directed mutagenesis to improve the enzyme for the synthesis of β-lactam antibiotics.

Mutating βF57, which is at the bottom of the substrate binding pocket, led to reduced activity but, surprisingly, not to changes in V_s/V_h ratios. This indicates that although mutations on this position do influence the interaction with the acyl donor, they have a much smaller effect on the interaction of H_2O and 6-APA with the acyl-enzyme. Mutating the residues that are closer to the active-site serine, βF24 and αF146, yielded mutants that were changed with respect to both activity and interaction with the deacylating nucleophiles.

The above indicates that the relative rates of hydrolysis and synthesis can be modified by site-directed mutagenesis. The study described in this paper does not involve the mutagenesis of the catalytic residues, but of residues located in the substrate binding pocket. Few examples exist in which the ratio between hydrolysis and aminolysis was changed by changing the catalytic nucleophile. By replacing the active-site serine in subtilisin with a cysteine, a 10^4-fold increased V_s/V_h ratio compared to the wild-type was obtained, probably because of the higher reactivity of thioesters with amine nucleophiles compared to water [13]. In protease B of *Streptomyces griseus* an effective ligase was created by replacing the active-site serine by an alanine [14]. In this case, the histidine which normally serves as the general acid/base became the nucleophile and catalysis proceeded via an acyl-imidazole intermediate. However, catalytic activities were reduced 10^3–10^4 fold in subtilisin and protease B mutants. The increased V_s/V_h ratio in the penicillin acylase mutants is not accompanied by such a loss of activity and the kinetic effects are probably caused by more subtle changes in structure around the active site serine, influencing the geometry of the acyl-enzyme and the relative position of the competing nucleophiles.

From the mutants that were analysed, βF24A appeared to be the most interesting with respect to synthesis of antibiotics. Compared to the wild-type enzyme, the βF24A mutant had a higher V_s/V_h, an increased esterase/amidase activity, and exhibited reduced inhibition by PAA. These observations are in line with results described by You *et al.* who found that by using βF24A increased yields in the synthesis of cefprozil and cefadroxil could be obtained [15]. However, the kinetic properties of the mutant enzyme underlying the improved performance of βF24A were not investigated.

The βF24A mutant enzyme had an increased V_s/V_h both with 7-ADCA and 6-APA as compared to the wild-type caused by an increased $(V_s/V_h)_{max}$. The data indicate that in both enzymes, hydrolysis of the acyl-enzyme to which 6-APA is bound still takes place, in agreement with results described earlier for the wild-type enzyme [11,12]. This indicates that the increased V_s/V_h ratio in the βF24A mutant is not caused by a displacement of the deacylating water molecule from the active site, but that the microscopic rate constants for the deacylation reaction in the active site of the acyl-enzyme have been changed by the βF24A mutation. The rate-limiting step in the synthesis reaction is the acylation of the enzyme and it therefore cannot be determined whether the reactivity with 6-APA and 7-ADCA (V_s) has increased or that the reactivity with H_2O (V_h) has decreased or that both reactivities have changed but to a different extent. The deacylating water molecule in PA is probably bound by the backbone of βQ23, and may have changed position upon mutating the neighbouring βF24 residue [7]. The binding of 6-APA, however, is governed by interactions with αR145, αF146 and βF71 and may be less disturbed by mutations on position βF24. It therefore seems likely that the increased V_s/V_h is caused by a decrease in water reactivity (V_h) rather than a changed 6-APA reactivity (V_s). However, changes in the β-lactam binding site caused by the βF24A mutation cannot be excluded.

An interesting property of βF24A is its increased esterase/amidase ratio. In general the enzyme-catalysed hydrolysis of esters is faster than the hydrolysis of the corresponding amides due to the intrinsically lower stability of the ester bond [16]. The hydrolysis of amides therefore requires more catalytic power than hydrolysis of esters. Several mechanisms to fulfil this requirement have been suggested. One mechanism is to bind the substrate in such a way that the planar character of the amide bond is disturbed. In this way substrate binding energy is used to change the structure of the peptide bond towards a structure that resembles the transition state [17]. The distortion may be achieved by interactions of the carbonyl oxygen with the residues in the oxyanion hole [18] or by interactions between the enzyme and the leaving group of the substrate [16,17]. Another mechanism involves the positioning of the catalytic base in such a way that it facilitates protonation of the leaving group [19]. The structural features responsible for the relatively high amidase activity encountered in PA are not known. The wild-type has a higher esterase/amidase ratio for phenylacetylated substrates than βF24A, whereas βF24A has a higher esterase/amidase ratio for phenylglycylated substrates (Table 3). This indicates that not only enzymatic properties but also substrate structural features play a role in determining the relative esterase/amidase activities of an enzyme. Crystallographic studies may provide more insight into the structural features underlying the kinetic properties of these enzymes.

The reduced amidase activity of βF24A influences the factor α, a key parameter for the synthesis of antibiotics [4,6]. It has been calculated that improvements of α below a value of 0.1 cause practically no extra yield in synthesis. The α values of βF24A are between 0.4 and 2, when the esters are used as acyl donors. Although this is a 10-fold improvement compared to the wild-type, the yield in antibiotic synthesis may still be further improved by decreasing α in this mutant.

It has been argued that PA is optimized in evolution for the conversion of phenylacetylated substrates [20]. This is confirmed by the results described in this paper that suggest that specificity of N-phenylacetylated substrates is difficult to improve by mutagenesis, because all mutants showed a decreased specificity for NIPAB and reduced affinity for PAA. The synthesizing capacity, e.g. interaction with the β-lactam nucleophile, is more easily improved, indicated by the values for V_s/V_h which were in almost all cases similar or higher than the wild-type values. Similar results have been obtained in a study in which other mutants were analysed (W. B. L. Alkema & D. B. Janssen, unpublished data). Site-directed mutagenesis seems therefore a promising way to improve penicillin acylase for biocatalytic application since this is not a function for which the enzyme has been optimized by evolution.

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